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Differences and overlap in plasma protein expression during colorectal cancer progression

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Abstract

Background: Colorectal cancer (CRC) is the third most common cancer worldwide, and its incidence is expected to increase to over 2.2 million new cases in 2030. Stage II CRC is classified as localized disease, while stage III CRC has spread to regional lymph nodes. The 5-year survival rate is over 80% for patients with stage II CRC, but less than 60% for patients with stage III CRC. Proteins, especially plasma proteins that are detectable in easily obtained blood samples, that differ between stage II and III CRC could be useful for predicting and monitoring disease progression. CRC displays differences depending on primary tumor location (right colon, left colon, or rectum), and how plasma protein expression changes during CRC progression from stage II to III depending on primary tumor location is not well-characterized.

Methods: In this study, we have used Ultra Performance Liquid Chromatography-Ultra Definition Mass Spectrometry (UPLC-UDMS^E)-based proteomics to analyze plasma samples from 83 patients with stage II or III CRC, followed by statistical and pathway analysis (data are available via ProteomeXchange). The patients were divided into groups according to tumor stage (II or III) and changes in plasma protein expression between stage II and III (localized and regional disease) samples were studied both regardless of primary tumor location and also within each primary tumor location (right colon, left colon, rectum).

Results: We discovered differences in plasma protein expression within all groups analyzed and identified proteins whose levels changed in one, two, or all three primary tumor locations between stage II and III CRC. Proteins were identified that could separate the groups compared and pathway analysis by IPA discovered altered pathways involved in lipid metabolism and inflammation, among others.

Conclusions: Plasma protein expression changes significantly as CRC progresses from stage II to III. While the levels of certain plasma proteins changed during cancer progression in only one or two primary tumor locations, the levels of 13 proteins changed in all primary tumor locations and are therefore common to CRC progression.

Keywords: Colorectal cancer, Proteomics, Plasma, Mass spectrometry, Stage, Cancer progression

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Background

Each year, over 1.4 million new cases of colorectal cancer (CRC) are diagnosed, making it the third most common cancer worldwide [1]. In addition, the CRC burden has been predicted to increase by 60% by 2030 [2]. While the 5-year survival rate is more than 60% in countries such as the United States and Switzerland, it ranges from 28 to 42% in developing countries [3]. There are also large differences in survival according to stage of disease – when CRC is detected at an early stage, the 5-year survival rate can be as high as 90%, but when the disease has spread to distant organs, it is only 12.5% [4, 5]. According to the TNM classification of malignant tumors, stage II CRC includes tumors with no regional lymph node metastasis. Stage III CRC is characterized by regional lymph node metastasis in any number of regional lymph nodes [6]. The TNM classification is anatomic-based and, although widely used, does not take into account additional patient or disease characteristics known to affect survival in CRC, such as age, gender, and primary tumor location. In patients with stage II and III CRC, the TNM system struggles to clearly distinguish groups of patients with different prognosis, especially in patients who receive adjuvant chemotherapy [7]. For stage II CRC patients the 5-year survival rate is 82.5%, and for stage III patients the 5-year survival rate is 59.5% [8].

Differences between tumors arising in the right and left colon have been well documented, and it has been suggested that right- and left-sided colon cancer should be considered to be separate entities [9–11]. Differences exist in embryologic origin, gross macroscopic pathology, and clinical presentation, with right-sided colon cancer typically presenting at a more advanced stage. Right-sided colon cancer has also been found to be more frequent in older people and women and to have a poorer prognosis than left-sided colon cancer [12, 13]. While colorectal cancer includes cancer both in the colon and rectum, rectal cancer differs in several ways from cancer in the colon, such as having a greater risk of local recurrence [14, 15]. Rectal cancer also tends to more frequently display mutations in genes such as *TP53* than colon cancer [15, 16]. How plasma protein expression changes during CRC progression from stage II to III depending on primary tumor location is something that has not previously been studied, as far as we know.

Mass spectrometric proteomic analysis is widely used to analyze the levels of hundreds of plasma proteins simultaneously and in efforts to elucidate molecular features of diseases at the protein level. The blood proteome is dynamic and reflects the state of the host due to the

perfusion of organs, therefore also reflecting the presence of diseases such as cancer. Biomarkers detectable from blood samples are useful due to the ease of sample collection [17, 18]. Previous proteomic studies of CRC have mainly attempted to find new biomarker candidates for the early detection of CRC and have compared samples from CRC patients and healthy controls. [19–21]. However, a study by Surinova et al. discovered different plasma protein signatures able to predict factors such as prognosis, regional tumor localization, and disease dissemination [22]. Few studies have used samples from cancer patients only, without the addition of healthy controls, although we have previously analyzed serum protein expression in a small set of CRC patients with varying C-reactive protein levels and survival times [23]. At the time of diagnosis in clinical settings, discerning cancer from healthy controls is not a requirement for differential diagnosis. Here, differences between various stages of cancer or prognostic determination become more important.

In this study, we have used Ultra Performance Liquid Chromatography-Ultra Definition Mass Spectrometry (UPLC-UDMS^E)-based proteomics to analyze plasma samples from CRC patients. The aim of this study was to study if there were observable differences in plasma protein expression between stage II and III CRC patients. This study provides new insights into how plasma protein expression differs during CRC progression, both regardless of and depending on primary tumor location.

Methods

Patient samples

This study used preoperative plasma samples from a total of 84 CRC patients, of which 37 were male and 47 female. This study included patients with stage II or stage III CRC who underwent surgical resection with curative intent in the Department of Surgery, Helsinki University Hospital, between 2000 and 2007. Plasma samples were stored at -80°C until processed as described below. We deliberately excluded patients with other cancers, a previous history of HNPCC, FAP, ulcerative colitis, Crohn's disease, or mucinous tumors from this study. Detailed patient characteristics are given in Additional file 1. The clinical data was obtained from patient records, the survival data from the Population Register Centre of Finland, and the cause of death for all the deceased from Statistics Finland. Written informed consent was obtained from all patients prior to collecting samples. This study conformed to the standards set by the Declaration of Helsinki and was approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013).

Sample processing and digestion

The plasma samples were processed as follows. The plasma samples were first thawed, after which top 12 protein depletion was carried out using the TOP12 protein depletion kit (Pierce, ThermoFisher, MA, USA) according to the manufacturer's instructions. The Pierce BCA assay kit (Pierce, ThermoFisher, MA, USA) was used to determine the protein concentration in each sample, and the amount of plasma equivalent to 100 µg of protein was aliquoted and dried using a SpeedVac (Savant, ThermoFisher, MA, USA). The dried plasma was dissolved in 35 µl Tris buffer (50 mmol/L, pH 7.8), which contained 6 M urea, and 1.8 µl of dithiothreitol (DTT, 200 mmol/L) was then added to each sample. The samples were shaken for 1 h at room temperature, and then 7 µl of iodoacetamide (200 mmol/L) per sample was added. Samples were shaken for 1 h at room temperature, and 7 µl of DTT (200 mmol/L) was added to each sample afterwards, and the samples were shaken for another hour at room temperature. The samples were then diluted with 270 µl mQ water per sample and trypsin was added at a ratio of 1:50 trypsin to protein. The samples were digested at 37 °C overnight, after which 30 µg of tryptic peptides were cleaned using C18 spin columns (Pierce, ThermoFisher, MA, USA). The cleaned peptides were then dissolved in 86 µl of 0.1% formic acid containing 12.5 fmol/µl of H13 spike-in standard peptides (Waters, MA, USA) for quantification.

Ultra performance liquid chromatography-ultra definition mass spectrometry and quantification

UPLC-UDMS^E

For UPLC-UDMS^E, four µl of each sample, equivalent to ~1.4 µg total protein, was injected to a nanoACQUITY UPLC system (Waters Corporation, MA, USA). TRIZAIC nano-Tile 85 µm × 100 mm HSS-T3u wTRAP was used as a separation device. Samples were loaded, trapped, and washed for two minutes with 8.0 µl 1% B and the analytical gradient used was as follows: 0–1 min 1% B, at 2 min 5% B, at 65 min 30% B, at 78 min 50% B, at 80 min 85% B, at 83 min 85% B, at 84 min 1% B, and at 90 min 1% B with 450 nL/min. Buffer A was 0.1% formic acid in water while buffer B was 0.1% formic acid in acetonitrile. Data were acquired in data-independent acquisition fashion using UDMSE mode with a Synapt G2-S HDMS (Waters Corporation, MA, USA). Calibration was performed with sodium iodide clusters over a mass range of 50–2500 m/z by infusing 2 µg/µl sodium iodide solution in 50/50 2-propanol/water into the mass spectrometer. 10% of the samples were run in triplicate and the median coefficient of variation (%CV) of the dataset was 4.36%.

Data analysis

Data analysis and label-free quantification were performed as previously described [23–25]. In summary, the raw files were imported to Progenesis QI for proteomics (Nonlinear Dynamics, Newcastle, UK). Post-acquisition mass correction was done when the raw data was imported into Progenesis with a lock mass ion of M + H + 556.2771 m/z. Leucine enkephalin (C25H37O7, 1 ng/µl in 50:50 acetonitrile:water + 0.1% formic acid) was infused into the reference sprayer at 300 nl/min for this purpose.

Default parameters were used for peak picking and alignment, while the peptide identification was done against Uniprot human FASTA sequences (release 2018_04). A ClpB protein sequence (CLPB_ECOLI (P63285)) was inserted for label-free quantification. Fixed modification at cysteine (carbamidomethyl) and variable at methionine (oxidation) were used. Trypsin was used as a digesting agent, with one missed cleavage allowed. Fragment and peptide error tolerances were set to automatic settings, and the false discovery rate (FDR) was set to less than 2%. For ion matching, one or more ion fragments per peptide, three or more fragments per protein, and one or more peptides per protein were required, which are the default parameters.

The parsimony principle was used to group the proteins and peptides unique to the protein were also reported. Progenesis QI for proteomics does not follow a strict parsimonious approach due to over-stringency, something that has been previously noted [26]. In the case of a conflict where two proteins were found with common peptides, the protein with fewer peptides was absorbed into the protein with more peptides. All relevant proteins are listed as a group under the lead protein with the highest coverage or score if the coverage of two or more proteins are equal. Quantification was performed using the lead identity peptide data. Further details can be found on the Nonlinear Dynamics' website (www.nonlinear.com).

Further analysis

The differences between the groups were analyzed using the Mann-Whitney U test. Principal Component Analysis (PCA) was performed using Progenesis QI for proteomics in order to help visualize the differences between the groups. Further analysis included Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modeling, used to classify the proteins expressed in the different groups, from which an S-plot was generated. All proteins were used as input for OPLS-DA modeling, but the passing criteria were a p (corr) cutoff value of greater than ±0.6 and a loading (p [1]) of between ±0.1. These S-plot criteria-passing proteins also had to pass the cutoff of a Mann-Whitney U test *p*-value of less than 0.05 for added stringency. Pathway

analysis was performed using Ingenuity Pathway Analysis (IPA) (QIAGEN Bioinformatics, Redwood City, CA) and the String 11.0 Database (accessed at <https://string-db.org>). Only proteins that passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis. The settings used for String pathway analysis were highest confidence and the active interaction sources used were “Experiments” and “Databases”. All other active interaction sources were excluded.

Results

Protein identification and comparisons

In this study, we analyzed plasma samples from 84 CRC patients that were divided into groups depending on tumor stage. One sample was not successfully digested and was subsequently excluded from further analysis, giving a total of 83 CRC patients, of which 34 had stage II CRC and 49 had stage III CRC. The patients included in this study are given and the excluded sample is marked in Additional file 1. We quantified a total of 224 proteins containing two or more unique peptides, and these proteins were used for further analysis. Proteins with p -values greater than 0.05 were not considered to be significantly different. The full list of proteins identified with relevant data is given in Additional file 2.

The plasma samples in this study were divided into different groups according to tumor stage (II or III), as well as according to tumor stage and location in the colon (right colon, left colon, rectum). The plasma samples in this study were therefore analyzed and the protein expression compared between all stage II and III CRC samples as well as between samples from patients with stage II and III cancer in the right colon, left colon, or rectum separately. Samples were also compared between stage II and III cancer in all primary tumor locations together. Proteins with p -values of less than 0.05 were considered to have significantly different levels between the groups.

Comparison of protein expression between patients with stage II and III CRC

When plasma samples from all stage II ($n = 34$) and stage III ($n = 49$) CRC patients were compared (regardless of primary tumor location), a total of 79 proteins passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 (Additional file 3 A). Of these proteins, 77 displayed higher levels in patients with stage II CRC, indicating that their concentrations decreased as CRC progressed from stage II to III. Only two proteins, MORC family CW-type zinc finger protein 2 (MORC2) and Filamin-A-interacting protein 1 (FILIP1), had higher levels in patients with stage III cancer, indicating that these

two proteins may be linked to disease progression and regional metastasis.

The PCA biplots showing all proteins (with two or more unique peptides) and, separately, only those proteins that passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 did not show a separation between the two groups. OPLS-DA modeling and the S-plot generated from this model identified one protein that passed all three criteria mentioned previously, which was Histidine protein methyltransferase 1 homolog (METTL18). METTL18 displayed higher levels in patients with stage II CRC and is given with its relevant data in Additional file 4 A.

Pathway analysis by IPA found multiple canonical pathways that were enriched in this dataset, such as LXR/RXR and FXR/RXR activation, the complement system, and acute phase response signaling. Pathway analysis by IPA also generated networks of protein-protein interactions, and the top network was found to be associated with the following functions: humoral immune response, inflammatory response, and developmental disorder (Additional file 5).

Comparison of protein expression between patients with right-sided stage II and III CRC

When samples from patients with right-sided stage II ($n = 11$) and stage III ($n = 16$) CRC were compared, a total of 97 proteins passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 (Additional file 3B). Two of these proteins, MORC2 and Ephrin type-A receptor 5 (EPHA5), displayed higher levels in samples from patients with right-sided stage III colon cancer. Levels of the other 95 proteins were higher in patients with stage II cancer in the right colon, indicating a decrease in expression from stage II to III cancer in the right colon. MORC2 displayed the largest fold change (5.7) of all 97 proteins in this group, with higher levels in samples from patients with stage III cancer in the right colon.

The PCA biplot when all proteins were considered showed a partial separation between samples from patients with right-sided stage II and III colon cancer (Fig. 1a). When only proteins with a Mann-Whitney U test p -value of less than 0.05 were considered, the separation became clearer, which was expected due to considering only differentially expressed proteins (Fig. 1b). OPLS-DA modeling and the S-plot generated from this model identified 21 plasma proteins that passed all three criteria mentioned previously (a p (corr) cutoff value of greater than ± 0.6 , a loading (p [1]) of between ± 0.1 , and a Mann-Whitney U test p -value of less than 0.05) and could discriminate between samples from patients with right-sided stage II and III colon cancer. The S-plot is shown in Fig. 1c and the 21 proteins are given in Table 1. The S-plot criteria-passing protein with the largest fold change (5.7) was MORC2, which had higher levels in

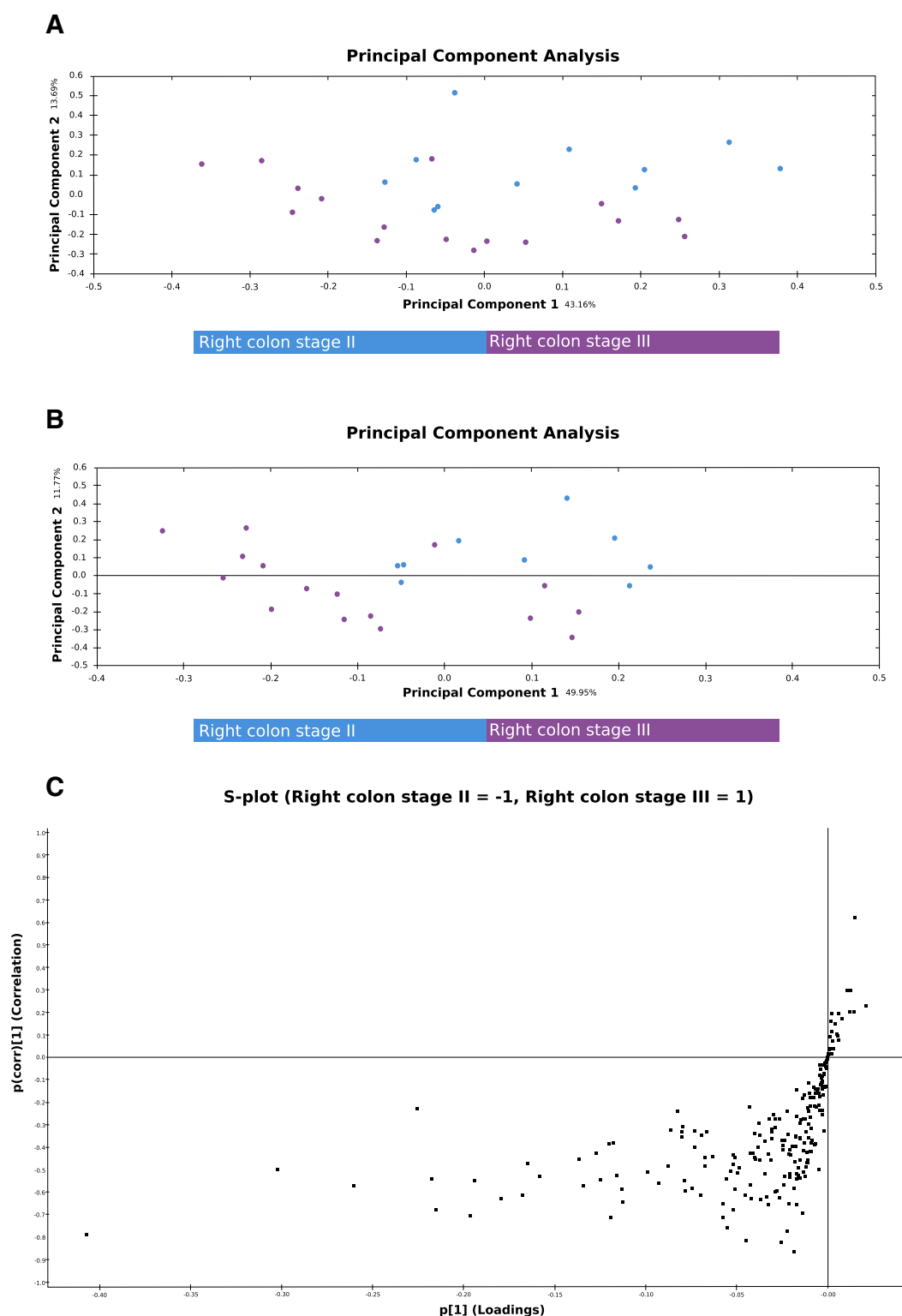


Fig. 1 Separation between proteins expressed in samples from patients with right-sided stage II and III CRC. **a** shows the PCA biplot when all proteins were considered **b** shows the PCA biplot when only proteins that passed the cutoff of a Mann-Whitney U test p-value of less than 0.05 were considered. Blue dots represent samples from patients with stage II cancer in the right colon and purple dots represent samples from patients with stage III cancer in the right colon. **c** shows the S-plot generated by OPLS-DA modeling. The upper right quadrant of the S-plot, with positive values, shows proteins with higher levels in samples from patients with right-sided stage III CRC. The lower left quadrant, with negative values, shows proteins with higher levels in samples from patients with right-sided stage II CRC

Table 1 The S-plot proteins differing between samples from patients with right-sided stage II and III CRC

Accession	Peptide count	Unique peptides	Confidence score	Mann-Whitney U test p-value	Fold change (right colon stage II/right colon III)	Protein name	Gene name	<i>p</i> [1]	<i>p</i> (corr) [1]
O95568	8	3	32,4	4,37E-05	1,6	Histidine protein methyltransferase 1 homolog	METTL18	-0,02	-0,86
P09429	5	2	29,5	1,43E-04	1,8	High mobility group protein B1	HMGB1	-0,03	-0,82
P68032; P62736; P68133; P63267	21	3	208,1	1,66E-03	1,8	Actin_ alpha cardiac muscle 1	ACTC1	-0,04	-0,82
P01344	7	4	27,5	1,66E-03	1,4	Insulin-like growth factor II	IGF2	-0,02	-0,77
Q96KN2	19	15	128,0	3,08E-03	1,4	Beta-Ala-His dipeptidase	CNDP1	-0,06	-0,76
P04083	17	8	99,3	1,95E-03	1,4	Annexin A1	ANXA1	-0,06	-0,71
Q9Y4B5	4	2	19,1	8,64E-04	1,9	Microtubule cr s-linking factor 1	MTCL1	-0,01	-0,69
H7BZ55	9	6	52,9	8,35E-03	1,4	Putative ciliary rootlet coiled-coil protein 2	CROCC2	-0,05	-0,68
Q562R1	12	4	95,6	2,28E-02	1,2	Beta-actin-like protein 2	ACTBL2	-0,02	-0,66
P00740	13	10	71,8	6,14E-04	1,4	Coagulation factor IX	F9	-0,03	-0,65
Q6UB98	6	2	36,6	4,79E-03	1,5	Ankyrin repeat domain-containing protein 12	ANKRD12	-0,06	-0,65
H3BMM5	3	2	13,7	1,20E-03	1,4	Uncharacterized protein		-0,02	-0,65
P02775	9	7	66,0	8,00E-05	1,5	Platelet basic protein	PPBP	-0,04	-0,63
Q14520	24	18	158,5	1,09E-02	1,4	Hyaluronan-binding protein 2	HABP2	-0,04	-0,63
P23508	5	3	27,9	1,09E-02	1,2	Colorectal mutant cancer protein	MCC	-0,03	-0,63
A5A3E0; Q6S8J3; P0CG39; Q9BYX7; E7EQ34; O14653	52	14	289,7	1,73E-04	1,4	POTE ankyrin domain family member F	POTEF	-0,03	-0,62
Q15166	6	2	62,1	1,24E-02	2,9	Serum paraoxonase/lactonase 3	PON3	-0,05	-0,61
O75882	16	12	98,9	4,92E-02	1,2	Attractin	ATRN	-0,03	-0,60
P35858; Q2M5E4	34	28	275,0	2,03E-02	1,4	Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	-0,08	-0,60
P43251	11	7	75,6	1,24E-02	1,5	Biotinidase	BTD	-0,03	-0,60
Q9Y6X9	5	3	27,1	6,14E-04	-5,7	MORC family CW-type zinc finger protein 2	MORC2	0,02	0,62

These proteins passed the criteria for OPLS-DA modeling and also had a Mann-Whitney U test p-value of less than 0.05. Accession, peptide count, unique peptides, confidence score, p-value, fold change, protein name, gene name, covariance (*p* [1]), and correlation (*p* (corr) [1]) are given in the table

patients with right-sided stage III colon cancer. MORC2 was also the only one of the 21 proteins identified that showed higher plasma levels in samples from patients with stage III cancer in the right colon.

Pathway analysis by IPA found similar canonical pathways as in the analysis of all stage II and III samples to be enriched in this dataset (Additional file 6). The top network of protein-protein interactions also generated by IPA is shown in Fig. 2a. This network was found to be associated with the following functions: hematological system development and function, organismal functions, and hematological disease (Additional file 7). Pathway analysis was also performed using the String database, and the protein-protein interaction network generated for this dataset is shown in Fig. 2b. This network shows that many of the proteins identified interact directly with each other in various ways. String shows direct connections linking the proteins used as input, whereas IPA fills in the gaps by adding proteins not detected in this study that are known to interact with the proteins detected.

Comparison of protein expression between patients with left-sided stage II and III CRC

The plasma samples from patients with left-sided stage II ($n = 10$) and stage III ($n = 16$) colon cancer were

analyzed separately. A total of 38 proteins passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 (Additional file 3C). Of these proteins, 10 displayed higher levels in the plasma of patients with stage III compared to stage II cancer in the left colon, showing that their concentrations in plasma increase as CRC progresses. The remaining 28 proteins displayed lower levels in left-sided stage III colon cancer, showing a decrease in plasma levels from stage II to III. The protein with the largest fold change observed (8.5) was Serum amyloid A-1 protein (SAA1), which displayed higher levels in patients with stage II cancer.

The PCA biplots when all proteins were considered and when only proteins with a Mann-Whitney U test p -value of less than 0.05 were considered did not show a separation between the groups. OPLS-DA modeling and the S-plot generated from this model identified 7 proteins (shown in Additional file 4B) that passed all three criteria mentioned previously (figures not shown). These proteins could therefore separate samples from patients with stage II and stage III cancer in the left colon. All 7 proteins identified showed higher plasma levels in patients with stage II cancer in the left colon, with Keratin, type I cytoskeletal 10 (KRT10) having the largest fold change (1.7).

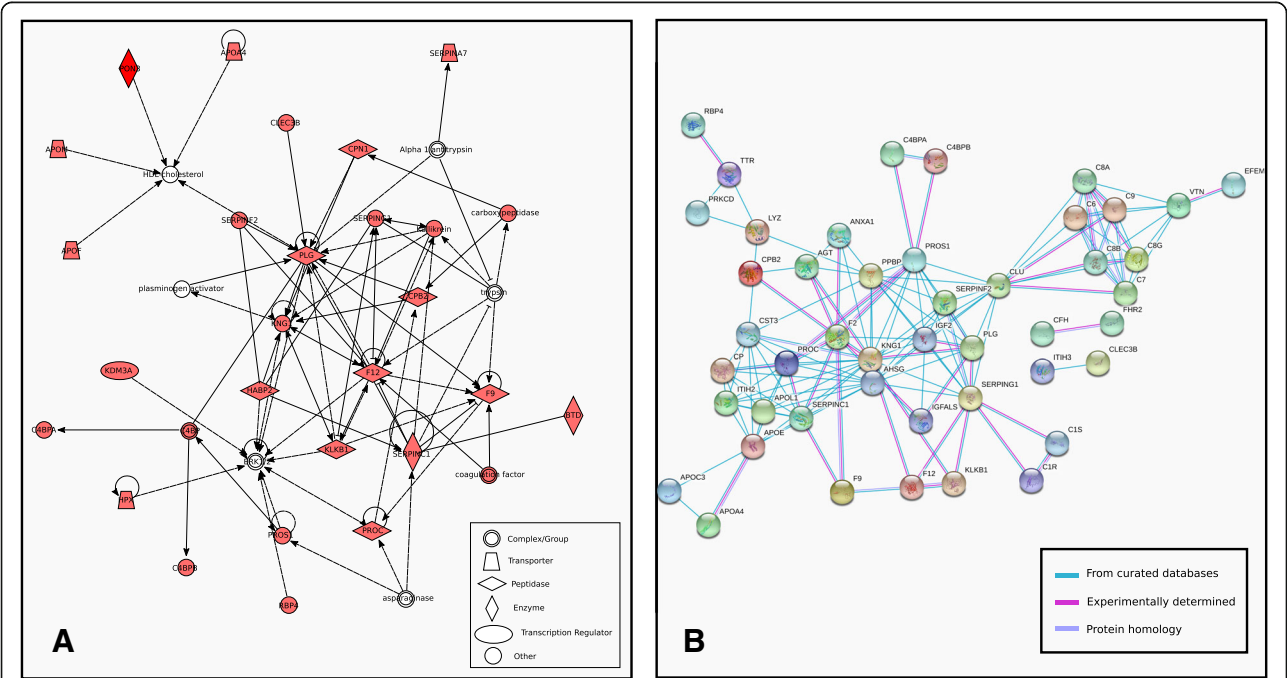


Fig. 2 Pathway analysis for samples from patients with right-sided stage II and III CRC. **a** shows the top protein-protein interaction network generated by IPA. Proteins in red are proteins that were identified in our study and which displayed higher levels in patients with stage II CRC in the right colon when compared to patients with stage III CRC in the right colon. Proteins in white are proteins that, although not identified in our study, play roles in this network and interact with the proteins identified. **b** shows the protein interaction network generated by String analysis. The network shows known, direct protein-protein interactions from experiments and databases, without the addition of intermediate proteins not detected in this study, which IPA adds. Only proteins that passed the cutoff of having a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis

Pathway analysis by IPA found similar canonical pathways to be enriched in this dataset as in the previous groups, although fewer pathways were found. The top network of protein-protein interactions generated by IPA was found to be associated with the following functions: dermatological diseases and conditions, developmental disorder, and hereditary disorder (Additional file 8).

Comparison of protein expression between patients with stage II and III rectal cancer

The plasma samples from patients with stage II ($n = 13$) and stage III ($n = 17$) rectal cancer were also analyzed separately. A total of 83 proteins passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 (Additional file 3D). Only two proteins (Extracellular superoxide dismutase [Cu-Zn] (SOD3) and C-reactive protein (CRP)) displayed higher levels in samples from patients with stage III rectal cancer. Levels of the other 81 proteins were all lower in samples from patients with stage III rectal cancer. The protein with the largest fold change (9.3) was Serum paraoxonase/lactonase 3 (PON3). PON3 displayed higher plasma levels in patients with stage II rectal cancer.

The PCA biplots when all proteins were considered and when only proteins with a Mann-Whitney U test p -value of less than 0.05 were considered did not show a clear separation between the groups. OPLS-DA modeling and the S-plot generated from this model (data not shown) identified a total of 24 proteins (shown in Additional file 4C) that passed all three criteria previously mentioned and which were therefore able to differentiate between samples from patients with stage II and III rectal cancer. All 24 proteins identified here displayed higher levels in stage II rectal cancer, with Keratin, type I cytoskeletal 16 (KRT16) having the largest fold change (8.4).

Pathway analysis by IPA found similar canonical pathways as in the previous analyses to be enriched in this dataset. The top network of protein-protein interactions generated by IPA was found to be associated with the following functions: humoral immune response, inflammatory response, and developmental disorder (Additional file 9).

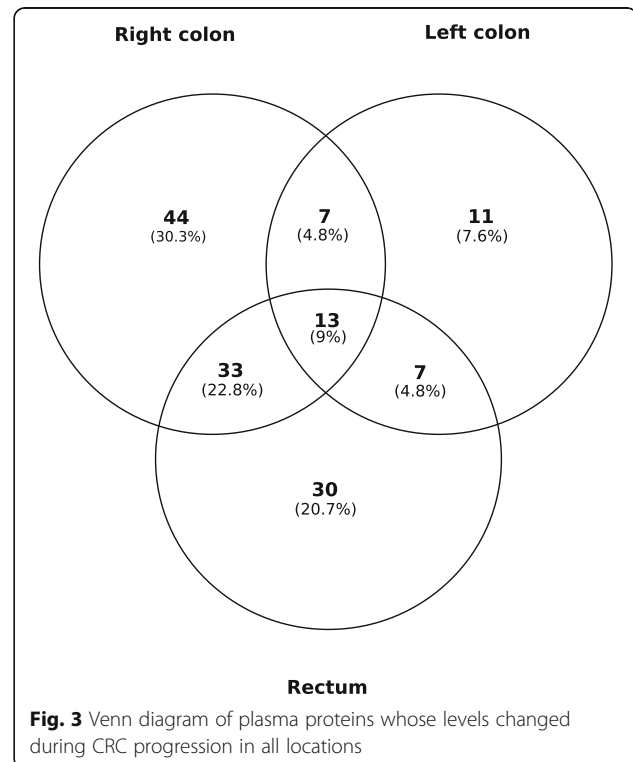
Venn diagram analysis for comparison of protein expression between primary tumor locations during cancer progression

In order to compare the protein expression between all three different primary tumor locations during cancer progression, we compared the proteins that significantly differed (those with a Mann-Whitney U test p -value of less than 0.05) between stage II and III CRC in each primary tumor location (right colon, left colon, or rectum)

with each other. In other words, we compared the proteins that passed the cutoff in the groups right colon stage II and III (97 proteins, found in Additional file 3B), left colon stage II and III (38 proteins, Additional file 3C), and rectum stage II and III (83 proteins, Additional file 3D) against each other. The Venn diagram in Fig. 3 shows how the proteins that differ between stage II and III CRC overlap between different primary tumor locations, and each circle contains the total number of proteins that significantly differed between stage II and III CRC in that primary tumor location. The proteins that overlap between primary tumor locations indicate changes in plasma protein expression that are common to two or more locations during cancer progression.

The figure shows the number of proteins whose expression differs between samples from patients with stage II and III CRC and their relation to primary tumor location. Proteins whose expression differed between samples from patients with stage II and III CRC in one, two, or all three possible primary tumor locations were identified.

Levels of 44 plasma proteins differed between samples from patients with stage II and III cancer in the right colon only (Additional file 10A), while levels of 11 proteins differed between samples from patients with stage II and III cancer in the left colon only (Additional file 10B). Levels of 30 proteins differed between samples from patients with stage II and III rectal cancer only (Additional file 10C). There were



33 proteins whose levels differed between samples from patients with stage II and III cancer both in the right colon and rectum (Additional file 11A), while levels of 7 proteins differed between samples from patients with stage II and III cancer in both the right and left colon (Additional file 11B). Levels of 7 other proteins differed between samples from patients with stage II and III cancer in both the left colon and rectum (Additional file 11C). Levels of 13 proteins differed between samples from patients with stage II and III CRC regardless of primary tumor location. Changes in the levels of these 13 proteins were therefore common to CRC progression for all locations and are given in Table 2.

All of the 33 proteins whose levels differed between samples from patients with stage II and III cancer in both the right colon and rectum displayed higher levels in stage II cancer. Among the 7 proteins whose levels differed between samples from patients with stage II and III cancer in both the right and left colon, most displayed higher levels in stage II CRC, although 3 proteins displayed higher levels in samples from patients with stage III cancer in the left colon. Of the 7 proteins whose levels differed between samples from patients with stage II and III cancer in the left colon and rectum, all had higher levels in stage II CRC, except for one protein (Kinesin-like protein KIF16B) that displayed higher levels in samples from patients with stage III cancer in the left colon.

Levels of 13 proteins were significantly different between samples from patients with stage II and III CRC regardless of primary tumor location. Levels of all 13 proteins were higher in samples from patients with stage II than stage III cancer in the right colon and rectum, indicating a decrease in plasma levels as CRC progressed. Levels of four proteins were higher in samples from patients with stage III cancer in the left colon, indicating an increase as cancer progressed from stage II to III, while levels of the remaining 9 proteins were higher in stage II cancer in the left colon. The protein with the largest fold change in all three groups was PON3. Surprisingly, PON3 displayed higher levels in samples from patients with stage II cancer in the right colon and rectum, but higher levels in samples from patients with stage III cancer in the left colon.

Discussion

In this study, we discovered differences in plasma protein expression between stage II and III CRC patients, both regardless of and depending on primary tumor location, and also identified proteins whose levels changed in only one, two, or all three primary tumor locations between stage II and III CRC. These results are interesting as they show that stage II and III tumors in the right colon, left colon, or rectum lead to the expression of different proteins to such extent that these differences can be detected in plasma samples. While a study by

Table 2 The Venn diagram proteins whose levels differed during CRC progression in all locations

Proteins whose plasma levels differed between samples from patients with stage II and III cancer in all locations

Accession	Peptide count	Unique peptides	Confidence score	Protein name	Gene name	Notes
Q15166	6	2	62,1	Serum paraoxonase/lactonase 3	PON3	*
P02766	22	19	214,4	Transthyretin	TTR	
P02775	9	7	66,0	Platelet basic protein	PPBP	*
O95568	8	3	32,4	Histidine protein methyltransferase 1 homolog	METTL18	
Q12805	9	8	51,7	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	
Q8TE85	6	3	28,7	Grainyhead-like protein 3 homolog	GRHL3	
P05455	5	3	28,9	Lupus La protein	SSB	*
A6NMZ7;P51570;Q13158;Q6NT55	5	2	24,3	Collagen alpha-6(VI) chain	COL6A6	
P09871	54	47	387,7	Complement C1s subcomponent	C1S	
O75882	16	12	98,9	Attractin	ATRN	
P23508	5	3	27,9	Colorectal mutant cancer protein	MCC	*
P02790;P51687;Q00973;Q04917;Q5T013;Q9NXR7;Q9NZ08;Q9Y2H2;Q9Y2K5;V9GYY9	182	150	1011,0	Hemopexin	HPX	
P07357	35	34	217,4	Complement component C8 alpha chain	C8A	

These 13 proteins are those whose changes in levels were common to CRC progression regardless of tumor location. Accession, peptide count, unique peptides, confidence score, protein name, and gene name are given in the table. The proteins with higher levels in samples from patients with left-sided stage III than stage II CRC are marked with a *. All other proteins had higher levels in patients with stage II cancer

Surinova et al. previously investigated differences in plasma protein expression between CRC tumors with different stage and location, their study focused more on biomarkers signatures comprised of several proteins rather than individual proteins [22].

In our current study, MORC2 displayed significantly higher levels in the plasma of stage III CRC patients, both when all stage II and III samples were compared and when only samples from the right and left colon were compared. MORC2 is a protein with roles in chromatin remodeling, facilitating DNA damage repair, and promoting lipogenesis. Mutations in MORC2 promote invasion and metastasis in triple-negative breast cancer [27]. MORC2 has also been shown to be upregulated in liver cancer and contribute to features such as proliferation and metastasis [28]. In lung cancer, MORC2 is also upregulated and enhances tumor growth by promoting angiogenesis [29]. In CRC, MORC2 has been shown to inhibit N-myc downstream regulated gene 1 (NDRG1), a metastasis suppressor, which promotes cell migration, invasion, and lung metastasis. This study also showed that MORC2 was upregulated in CRC tissues, and high expression was found to be associated with lymph node metastasis [30]. We found that MORC2 levels are higher in plasma samples from patients with lymph node positive CRC, and the findings of these studies together indicate that MORC2 is of interest as a new biomarker, although further studies are needed to elucidate its role in CRC.

Here, we also identified plasma proteins whose expression changed between stage II and III cancer in one, two, or all three primary tumor locations. Levels of certain plasma proteins changed between stage II and III cancer only in the right colon, left colon, or rectum. Surprisingly, there were more proteins that were common between cancer in the right colon and rectum (33 proteins) than between cancer in the left colon and rectum (7 proteins), even though the right colon and left colon are anatomically more similar and the left colon and rectum are anatomically closer to each other. Levels of 13 proteins changed between samples from patients with stage II and III cancer in all primary tumor locations. These 13 proteins were therefore common to stage II to III cancer progression and could be of use in predicting disease progression from local to regional disease, although further validation is needed.

Pathway analysis by IPA identified LXR/RXR activation and FXR/RXR activation as the two most enriched pathways both when all stage II and III samples were analyzed, as well as when stage II and III samples were analyzed separately between samples from the right colon, left colon, and rectum. LXRs are nuclear receptors that play important roles in cholesterol metabolism as well as in the biosynthesis and metabolism of very low-density

lipoproteins [31]. FXRs, also nuclear receptors, regulate bile acid synthesis and various aspects of lipid and glucose metabolism. LXRs and FXRs both form heterodimers with RXRs [32, 33]. Lipid metabolic reprogramming occurs in cancer cells, with specific alterations in various aspects of lipid metabolism being seen [34, 35]. MORC2 has been found to interact with ATP citrate lyase (ACLY), which catalyzes the formation of acetyl-coenzyme A, which is important in lipid and cholesterol metabolism. In breast cancer cells, MORC2 promotes ACLY activation, giving MORC2 a role in lipogenesis [36]. PON3 is primarily localized to the high-density lipoprotein (HDL) fractions of plasma and is synthesized in the liver [37]. PON3 also possesses anti-inflammatory properties, and depletion of PON3 has been correlated with the pro-inflammatory activity of HDL [38]. Our findings that plasma levels of MORC2 and PON3 change between stage II and III CRC in various locations indicate that lipid metabolism is altered during CRC progression. The enrichment of lipid metabolism-associated pathways found by IPA further strengthens these findings.

Here, we observed changes in the levels of multiple plasma proteins between stage II and III CRC, both when all samples were compared and when samples were analyzed separately according to tumor location. These proteins could potentially be of use in predicting disease progression in a non-invasive way through blood sampling after further studies and large-scale validation. We discovered multiple plasma proteins whose levels only changed during cancer progression in the right colon, left colon, or rectum, as well as proteins whose levels changed in two or all locations. This further supports the notion that right- and left-sided CRC significantly differ from each other and that rectal cancer differs from colon cancer. The strengths of this study include the number of samples analyzed and the multiple groups between which comparisons were made. This study was limited by the lack of CRC tissue samples, as it would have been interesting to relate plasma protein expression to tissue protein expression. This study is one of very few studies showing that differences in plasma protein expression can be seen between patients with stage II and III CRC, both regardless of and within different primary tumor locations.

Conclusions

In this study, we found differences in plasma protein expression between patients with stage II and III CRC, both when samples were compared regardless of and according to primary tumor location. This is the first study to show that CRC progression from stage II to III has distinct, measurable effects on plasma protein expression and that plasma protein expression differs and overlaps during cancer progression depending on primary tumor location.

Additional files

Additional file 1: Information about the colorectal cancer patients included in this study. The table shows patients' gender, age at operation, tumor stage, location, and sample code. (XLSX 13 kb)

Additional file 2: The 224 proteins with two or more unique peptides quantified in this study. This table shows all proteins with two or more unique peptides that were quantified in this study. Accession, peptide count, unique peptides, protein name, gene name, and normalized abundance of each protein in each sample are given in the table. (XLSX 283 kb)

Additional file 3: The proteins that passed the cutoff of a Mann-Whitney U test p -value of less than 0.05 in the different groups. Additional file 3A shows the 79 proteins that passed the cutoff when all stage II and III samples were compared. Additional file 3B shows the 97 proteins that passed the cutoff when samples from patients with right-sided stage II and III CRC were compared, and Additional file 3C shows the 38 proteins that passed the cutoff when samples from patients with left-sided stage II and III CRC were compared. Additional file 3D shows the 83 proteins that passed the cutoff when samples from patients with stage II and III rectal cancer were compared. Accession, peptide count, unique peptides, confidence score, p -value, fold change, protein name, and gene name are given in the tables. (XLSX 40 kb)

Additional file 4: The S-plot proteins that passed the criteria for OPLS-DA modeling and also had a Mann-Whitney U test p -value of less than 0.05. Additional file 4A shows the one protein that passed all three criteria when all stage II and III samples were compared, and Additional file 4B shows the 7 proteins that passed all three criteria when samples from patients with left-sided stage II and III CRC were compared. Additional file 4C shows the 24 proteins that passed all three criteria when samples from patients with stage II and III rectal cancer were compared. Accession, peptide count, unique peptides, confidence score, p -value, fold change, protein name, gene name, covariance (p [1]), and correlation (p (corr) [1]) are given in the tables. (XLSX 14 kb)

Additional file 5: The results of IPA pathway analysis when all samples from patients with stage II and III CRC were compared. This file shows the results of Ingenuity Pathway Analysis when all stage II and III samples were compared. It includes information about the top canonical pathways enriched and the protein-protein networks enriched and their associated functions. Only proteins that passed the cutoff of having a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis. (PDF 86 kb)

Additional file 6: The canonical pathways enriched in the group of patients with right-sided stage II and III CRC. This figure shows the top canonical pathways enriched when samples from patients with right-sided stage II and III CRC were compared using Ingenuity Pathway Analysis. Only proteins that passed the cutoff of having a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis. (PDF 20 kb)

Additional file 7: The results of IPA pathway analysis when samples from patients with right-sided stage II and III CRC were compared. This file shows the results of Ingenuity Pathway Analysis when samples from patients with right-sided stage II and III CRC were compared. It includes information about the top canonical pathways enriched and the protein-protein networks enriched and their associated functions. Only proteins that passed the cutoff of having a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis. (PDF 83 kb)

Additional file 8: The results of IPA pathway analysis when samples from patients with left-sided stage II and III CRC were compared. This file shows the results of Ingenuity Pathway Analysis when samples from patients with left-sided stage II and III CRC were compared. It includes information about the top canonical pathways enriched and the protein-protein networks enriched and their associated functions. Only proteins that passed the cutoff of having a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis. (PDF 85 kb)

Additional file 9: The results of IPA pathway analysis when samples from patients with stage II and III rectal cancer were compared. This file shows the results of Ingenuity Pathway Analysis when samples from patients with stage II and III rectal cancer were compared. It includes

information about the top canonical pathways enriched and the protein-protein networks enriched and their associated functions. Only proteins that passed the cutoff of having a Mann-Whitney U test p -value of less than 0.05 were used for pathway analysis. (PDF 85 kb)

Additional file 10: The proteins whose levels differed between samples from patients with stage II and III CRC in one primary tumor location only. Additional file 10A shows the 44 proteins whose levels differed between stage II and III samples from patients with right-sided CRC only, Additional file 10B shows the 11 proteins whose levels differed between stage II and III samples from patients with left-sided CRC only, and Additional file 10C shows the 30 proteins whose levels differed between stage II and III samples from patients with rectal cancer only. Accession, peptide count, unique peptides, confidence score, protein name, and gene name are given in the tables. (XLSX 16 kb)

Additional file 11 The proteins whose levels differed between samples from patients with stage II and III CRC in two primary tumor locations. Additional file 11A shows the 33 proteins whose levels differed between stage II and III samples from patients with cancer in the right colon and rectum, Additional file 11B shows the 7 proteins whose levels differed between stage II and III samples from patients with cancer in the right and left colon, and Additional file 11C shows the 7 proteins whose levels differed between stage II and III samples from patients with cancer in the left colon and rectum. Accession, peptide count, unique peptides, confidence score, protein name, and gene name are given in the tables. (XLSX 14 kb)

Abbreviations

CRC: Colorectal cancer; IPA: Ingenuity Pathway Analysis; OPLS-DA: Orthogonal Projections to Latent Structures Discriminant Analysis; PCA: Principal Component Analysis; UPLC-UDMS^E: Ultra Performance Liquid Chromatography-Ultra Definition Mass Spectrometry

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Authors' contributions

MH, SJ, MS, AR, RR, and CH conceived and designed the study. MH, SJ, and TT acquired the data. MH, SJ, and MS analyzed and interpreted the data. MH wrote the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The mass spectrometry proteomics data supporting the conclusions of this article are available at the ProteomeXchange Consortium via the PRIDE [39] partner repository with the dataset identifier PXD013150 and <https://doi.org/10.6019/PXD013150>. Additional data supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate

Written informed consent was obtained from all patients prior to collecting samples. This study was approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 \$66 17.4.2013).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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